Enzyme-linked immunosorbent assay (ELISA). ELISA is an immunochemical technique that avoids the hazards of radiochemicals and the expense of fluorescence detection systems. Instead, the assay uses enzymes as indicators. ELISA is a form of quantitative immunoassay based on the use of antibodies (or antigens) that are linked to an insoluble carrier surface, which is then used to 'capture' the relevant antigen (or antibody) in the test solution. The antigen-antibody complex is then detected by measuring the activity of an appropriate enzyme that had previously been covalently attached to the antigen (or antibody).

For information on ELISA techniques, *see*, for example, J.R. Crowther, *Elisa: Theory and Practice (Methods in Molecular Biology*, Vol. 42), Human Pr. (1995); Challacombe and Kemeny, *ELISA and Other Solid Phase Immunoassays: Theoretical and Practical Aspects*, John Wiley & Son Ltd. (1998); D.M. Kemeny, *A Practical Guide to Elisa*, Pergamon Pr. (1991); and E. Ishikawa, *Ultrasensitive and Rapid Enzyme Immunoassay (Laboratory Techniques in Biochemistry and Molecular Biology*, V. 27), Elsevier Advanced Technology (1991).

Colorimetric Assays for Enzymes. Colorimetry is any method of quantitative chemical analysis in which the concentration or amount of a compound is determined by comparing the color produced by the reaction of a reagent with both standard and test amounts of the compound, often using a colorimeter. A colorimeter is a device for measuring color intensity or differences in color intensity, either visually or photoelectrically.

Standard colorimetric assays of beta-galactosidase enzymatic activity are well known to those skilled in the art (see, for example, Norton et al., Molecular & Cellular Biology 5:281-290 (1985)). A colorimetric assay can be performed on whole cell lysates using O-nitrophenyl-beta-D-galactopyranoside (ONPG, Sigma, St. Louis, Mo.) as the substrate in a standard colorimetric beta-galactosidase assay (Maniatis *et al.*, Cold Spring Harbor, N.Y., Cold Spring Harbor Lab. (1990)). Automated colorimetric assays are also available for the detection of beta-galactosidase activity, as described in U.S. Patent No. 5,733,720.

Immunofluorescence Assays. Immunofluorescence or immunofluorescence microscopy is a technique in which an antigen or antibody is made fluorescent by conjugation to a fluorescent dye and then allowed to react with the complementary antibody or antigen in a tissue section or smear. The location of the antigen or antibody

can then be determined by observing the fluorescence by microscopy under ultraviolet light.

For general information on immunofluorescent techniques, see, for example, Knapp *et al.*, Immunofluorescence and Related Staining Techniques, Elsevier/North-Holland Biomedical Press (1978); V.J. Allan, *Protein Localization by Fluorescent Microscopy: A Practical Approach* (The Practical Approach Series, 218), Oxford Univ. Press (1999); E.H. Beutner, *Defined Immunofluorescence and Related Cytochemical Methods*, New York Academy of Sciences (1983); and E.O. Caul, *Immunofluorescence Antigen Detection Techniques in Diagnostic Microbiology*, Cambridge Univ. Press (1993). For detailed explanations of immunofluorescent techniques applicable to the present invention, see, U.S. Patent Nos. 5,912,176; 5,869,264; 5,866,319; and 5,861,259.

O. Combinatorial Chemistry

Combinatorial chemistry can be utilized to generate compounds which are chemical variations of compounds useful in the present invention. Such compounds can be evaluated using the high-throughput screening methods of the present invention. Basic combinatorial chemistry concepts are well known to one of ordinary skill in the chemical arts and can also be found in Nicholas K. Terrett, Combinatorial Chemistry (Oxford Chemistry, Masters), Oxford Univ. Press (1998); Anthony W. Czarnik and Sheila Hobbs Dewitt (Editors), A Practical Guide to Combinatorial Chemistry, Amer. Chemical Society (1997); Stephen R. Wilson (Editor) and Anthony W. Czarnik (Contributor), Combinatorial Chemistry: Synthesis and Application, John Wiley & Sons (1997); Eric M. Gordon and James F. Kerwin (Editors), Combinatorial Chemistry and Molecular Diversity in Drug Discovery, Wiley-Liss (1998); Shmuel Cabilly (Editor), Combinatorial Peptide Library Protocols (Methods in Molecular Biology), Human Press (1997); John P. Devlin, High Throughput Screening, Marcel Dekker (1998); Larry Gold and Joseph Alper, Keeping pace with genomics through combinatorial chemistry, Nature Biotechnology 15, 297 (1997); Aris Persidis, Combinatorial chemistry, Nature Biotechnology 16, 691-693 (1998).

P. Modifying Thapsigargin, Cyclopiazonic Acid and DBHQ To Increase Therapeutic Efficacy

Thapsigargin, cyclopiazonic acid and 2,5-di-(*tert*-butyl)-1,4-hydroquinone (DBHQ) inhibit the ER Ca-ATPase, resulting in the transient elevation of cytosolic calcium levels and the depletion of ER calcium stores. While this activity underlies the proposed therapeutic benefit of these three compounds in CF, it is possible that it may also produce

toxic side effects by activating calcium-dependent processes in a wide variety of cells. Since the primary affected organ in CF is the lung, correction of the CF defect in airway epithelial cells would dramatically reduce the morbidity associated with this disease. It would be desirable, therefore, to construct derivatives of these compounds which could be applied locally to the airway by aerosol inhalation and which would not diffuse out of the airway epithelial cells to enter the systemic circulation. Such derivatives would be much less likely to exhibit systemic toxic side effects.

A non-specific esterase activity is present in the cytoplasm of most eukaryotic cell types. This activity has been exploited in the design of numerous compounds whose purpose is to enter the cytoplasm of target cells and subsequently remain trapped there. These compounds, which include several indicator dyes used to measure intracellular ionic concentrations, are synthesized as acetoxymethylesters (Grynkiewicz G, Poenie M and Tsien RY, A new generation of Ca indicators with greatly improved fluorescence properties, J. Biol. Chem. 260:3440-3450 (1985)). In this form they are membrane permeant and can diffuse across the cell membrane to enter the cytoplasm. The action of the cytoplasmic esterase removes methanol groups, leaving behind negatively charged carboxylic acid residues on the compound of interest. In this charged state, the compound is no longer membrane permeant and it is thus trapped in the cytosol.

Thapsigargin, cyclopiazonic acid and DBHQ may be modified to incorporate acetoxymethylester groups. These modified compounds would then be administered by aerosol inhalation. Presumably, they would enter the surface airway epithelial cells by diffusing across their apical plasma membranes. Once inside the airway epithelial cells, they would become substrates for the action of the cytoplasmic esterase. Esterase action on the derivatized compounds would leave these compounds with negatively charged carboxylic acid residues, thus preventing their departure from the airway epithelial cells. Consequently, the compounds would only gain access to and exert effects upon airway epithelial cells, which are their intended target. The potential for systemic side effects would thus be greatly reduced.

This strategy will succeed only if the addition of one or more carboxylic acid groups to thapsigargin, cyclopiazonic acid or DBHQ does not markedly reduce their inhibitory effects on the ER Ca-ATPase. No modifications may be necessary to reduce the toxicity of at least some of these compounds. Animal toxicity has not been associated with DBHQ (Chao *et al.*, Calcium- and CaMKII-dependent chloride secretion induced by the